MANNOSE TRANSFER TO LIPID LINKED DI-N-ACETYLCHITOBIOSE

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SUMMARY: Mannose was found to be transferred from guanosine diphosphate mannose to dolichol-P-P-di-N-acetylchitobiose when these substrates were incubated with hen oviduct or rat liver microsomes. The oligosaccharide moiety of the product appears to be the trisaccharide β-mannosyl-β-N-acetylglucosaminyl-(1→4)-N-acetylglucosamine as judged by the action of glycosydases. Under certain conditions further transfer of mannose occurs and larger oligosaccharides bound to lipid are formed.

The formation of lipid linked N-acetylglucosamine by transfer from UDP-GlcNAc to endogenous acceptor (1) or to dolichol monophosphate (2) has been found to be catalyzed by microsomal enzymes. The product formed has been compared with synthetic dolichol-P-P-GlcNAc and found to be the same by several criteria (3). This substance has been found to accept another N-acetylglucosamine on incubation with UDP-GlcNAc and liver microsomes giving rise to the formation of dolichol-P-P-di-N-acetylchitobiose (4). On the other hand it is known that a common constituent of many glycoproteins is β -mannosyl-di-N-acetylchitobiose linked to asparagine (5). These facts and the recent finding that transfer of mannose from GDP-Man to an acceptor lipid gives rise to the formation of mannose containing oligosaccharides bound to dolichol diphosphate (6), led to the postulation of a scheme of glycoprotein biosynthesis. According to this scheme dolichol-P-P-di-N-acetyl-chitobiose acts as acceptor for additional glycosyl residues until an oligosaccharide is formed which is then transferred to the protein acceptor (6,7). This mechanism of biosynthesis therefore differs from the one usually accepted (8) in that the glycosyl residues are transferred successively not to the glycoprotein but to dolichol

phosphate sugar. At present it appears that this mechanism is restricted to glycoproteins in which the oligosaccharide is linked to an asparagine residue.

This paper reports studies on the transfer of mannose from GDP-Man to dolichol-P-P-di-N-acetylchitobiose.

METHODS Substrates Dolichol-P-P-di-N-acetylchitobiose labelled in the hexosamine moiety was prepared by incubation of dolichol-P and microsomes first with labelled and afterwards with non labelled UDP-GlcNAc (4). It was purified by DEAE-cellulose chromatography (9). The inhibitor of β-N-acetylglucosaminidase (2-acetamido-2-deoxy-D-gluconolactone) was prepared according to Findlay et al. (10). Other substrates were described in previous papers (4,11).

Enzymes Hen oviduct and rat liver microsomes were prepared according to Moulé et al. (12). α-mannosidase from jack bean meal was prepared according to Li (13) up to the Bio Gel P-200 step. It was practically devoid of β-mannosidase but contained some β-N-acetylglucosaminidase (about one tenth as active). α-mannosidase from bromelain was prepared as described by Li and Lee (14) up to the ammonium sulfate step. The crude pineapple bromelain was kindly provided by Dole Co., Honolulu, Hawaii. β-N-acetylglucosaminidase from hen oviduct was prepared according to Sukeno et al. (14). The preparation was free from α and β-mannosidase. Saliva glycosidases were obtained by centrifuging and dialyzing human saliva. Tests were carried out under the conditions described by Levy and Conchie (15). Incubations were performed under toluene in order to prevent microbial growth.

Standard assay Dolichol-P-P-di-N-acetylchitobiose (about 1500 cpm labelled in the hexosamine), was dried in a test tube with 0.5 μmoles of Mg-EDTA and 0.5 μmoles of $MgCl_2$. The following reaction mixture was then added: 0.1 M mercaptoethanol, 0.1 M Tris-maleate (pH 7.8), 0.5% sodium deoxycholate, 3 mM GDP-Man and hen oviduct microsomes (about 2 mg of protein) in a total volume of 50 μl. After incubating for 4 hrs at 30°, the products were isolated by adding chloroform-methanol - 4 mM $MgCl_2$ (3:2:1) to the reaction mixture. The organic phase was separated, washed and dried, the lipids were then hydrolyzed and chromatographed on paper as described previously (4).

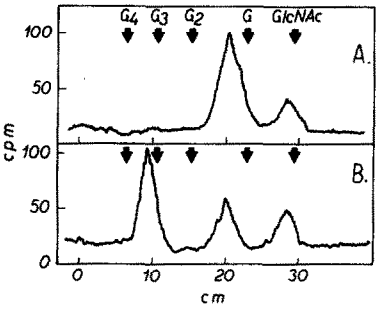


Figure 1 Paper chromatography (solvent A) of the products of mild acid hydrolysis of the lipid fraction corresponding to an incubation of radioactive dolichol-P-P-di-N-acetylchitobiose and unlabelled GDP-Man as indicated under Methods. A - control (non incubated) B - incubation with hen oviduct microsomes.

G = glucose, G_2 = maltose, etc.

Paper chromatography Solvent A: 1-butanol-pyridine-water (6:4:3); Solvent B, the same but the proportions were 4:3:4.

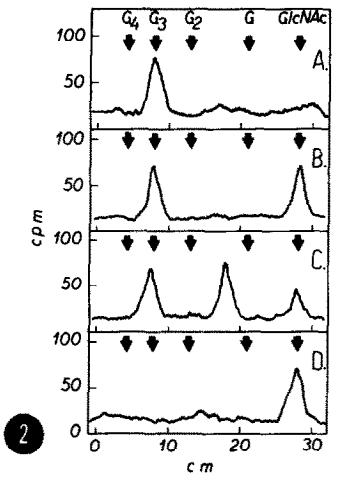
RESULTS Mannose transfer Incubation of radioactive dolichol-P-P-di-N-acetylchitobiose with unlabelled GDP-Man and hen oviduct microsomes led to changes in the sugar moiety of the former. These could be detected by mild acid hydrolysis followed by paper chromatography of the liberated saccharides. In Fig. 1 it can be seen that a slower moving substance was formed. It migrated (solvent A) between maltotriose and maltotetraose. This substance will be referred to as X_3.

Lipid bound X_3 was found to elute together with dolichol-P-P-di-N-acetylchitobiose on chromatography on DEAE-cellulose, so that the non oligosaccharide moiety is probably the same in both compounds.

Addition of dolichol-P to the reaction mixture did not increase the amount of lipid bound X_3 formed. Furthermore when dolichol-P-Man was substituted for GDP-Man, the amount of lipid bound X_3 formed was negligible.

Other experiments were carried out with the two substrates (GDP-Man

Abbreviations: X_3 = The oligosaccharide formed by addition of mannose to di-N-acetylchitobiose.



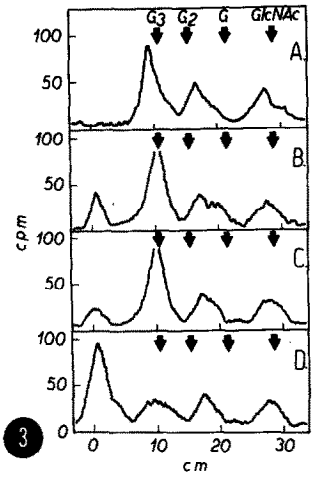


Figure 2 The action of glycosidases on X_3 (chromatography as in Fig. 1). A - control (non incubated.) B - incubated 25 hrs with β-mannosidase plus β-N-acetylglucosaminidase of human saliva. C - the same but in the presence of 2 mg of 2-acetamido-2-deoxyglucono lactone. D - incubated with β-N-acetylglucosaminidase from hen oviduct.

Figure 3 Incubation of labelled lipid bound X_3 with oviduct microsomes and: A - No addition. B - GDP-Man (0.3 μmole) and dolichol-P. C - GDP-Man. D - Dolichol-P-Man. Chromatography as in Fig. 1.

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and Dol-P-P-di-N-acetylchitobiose) labelled with ^{14}C. After extraction with chloroform-methanol, mild acid hydrolysis and paper chromatography a compound which migrated like X_3 was detected. This compound was eluted, hydrolyzed (6 hrs at 100° in 4 N HCl) and chromatographed on paper. The radioactivity was found in the same position as the glucosamine and the mannose standards.

The formation of X_3 is not restricted to hen oviduct since essentially the same results were obtained with rat liver microsomes.

The action of glycosidases Difficulties were experienced in the preparation of pure glycosidases particularly with β-mannosidase. Every preparation was checked for activity on p-nitrophenyl-α and β-mannosides and β-N-acetylglucosaminide.

The tests were carried out on X_3 obtained by mild acid hydrolysis followed by paper chromatography. The label was located at the reducing N-acetylglucosamine residue. Two preparations of α-mannosidase, one from bromelain and another from jack bean were found to have no action on X_3. These preparations were active in liberating mannose from labelled oligosaccharides obtained by transfer from radioactive GDP-Man to endogenous acceptors (5). A jack bean preparation containing α-mannosidase and β-N-acetylglucosaminidase was also found to be inactive on X_3.

Further tests were carried out with dialyzed human saliva. The relative activity of the enzymes was found to vary considerably in samples from different donors. A preparation which was very active in β-N-acetylglucosaminidase and with very weak α and β-mannosidases did not act on X_3. When incubated under the same conditions with di-N-acetylchitobiose it hydrolyzed it partially.

Another saliva preparation which contained active β-mannosidase and β-N-acetylglucosaminidase led to a considerable liberation of N-acetylglucosamine (Fig. 2 - B). The same experiment but with 2-acetamido-2-deoxygluconolactone, which is a strong inhibitor of β-N-acetylglucosaminidase, led to the liberation of di-N-acetylchitobiose (Fig. 2 - C). The latter was identified by chromatography in several solvents (4).

Incubation of X_3 with hen oviduct β-N-acetylglucosaminidase (free from α and β-mannosidases) led to the liberation of free N-acetylglucosamine. The same enzyme was tested on X_3 labelled in the mannose residue. The result was the formation of a substance which migrated like maltose (R_G = 0.63) on paper chromatography (solvent A). It could be distinguished from di-N-acetylchitobiose (R_G = 0.8) with other solvents also. Presumably this compound was β-mannosyl-N-acetylglucosamine.

The formation of higher oligosaccharides In order to find out if lipid bound X_3 can accept more mannose residues, experiments were carried out by incubating hen oviduct microsomes with dolichol-P, GDP-Man and labelled lipid bound X_3. The latter had been purified by DEAE-cellulose

chromatography and contained some dolichol-P-P-di-N-acetylchitobiose. After mild acid hydrolysis the oligosaccharide moiety was run on paper as described above. Compounds were found to be formed which remained at the origin when chromatographed with solvent A (Fig. 3 - B) and migrated with solvent B like maltooligosaccharides of 6 to 9 units. In experiments in which no dolichol-P was added the amount of higher oligosaccharides formed was much lower (Fig. 3 - C). Furthermore, when unlabelled dolichol-P-Man was substituted for GDP-Man, formation of higher oligosaccharides was also observed (Fig. 3 - D). In a previous paper similar compounds were detected after incubation of labelled GDP-Man with liver microsomes (5). The structure of these compounds is under investigation. Preliminary results indicate that they contain some mannose residues linked in α-configuration.

Discussion The results reported show that hen oviduct microsomes, as well as those of rat liver, catalyze the transfer of mannose from GDP-Man to dolichol-P-P-di-N-acetylchitobiose. The product formed is very similar to the latter since it is not separated by DEAE-cellulose chromatography. However mild acid hydrolysis leads to the liberation of a substance which appears to be a trisaccharide as judged by its mobility during paper chromatography. The putative trisaccharide was hydrolyzed by β but not by α-mannosidase. Saliva β-N-acetyl-glucosaminidase was inactive whereas that from hen oviduct, which has a different specificity, led to the liberation of N-acetylglucosamine. This enzyme has been found to hydrolyze a trisaccharide present in the linkage region of ribonuclease B which probably has a β-mannosyl on the 3 position of the non-reducing N-acetylglucosamine residue of di-N-acetylchitobiose (17).

Mannose transfer does not appear to take place with dolichol-P-Man as an intermediate since the addition of dolichol-P to the reaction mixture did not increase the yield of trisaccharide. Furthermore substitution of dolichol-P-Man for GDP-Man as donor resulted in no transfer. However, higher saccharides are formed by transfer from dolichol-P-Man to the trisaccharide containing lipid. Some of the mannose residues were α as would be predicted if each reaction occurs with inversion (6).

This trisaccharide formed from dolichol-P-P-di-N-acetylchitobiose is very similar or identical to the one found in egg ovalbumin (18). It seems to be the common structural core of the oligosaccharide joined to asparagine since it occurs in many glycoproteins (5).

The finding of the transfer reactions reported provides further evidence for the theory which postulates that core oligosaccharides of the asparagine type of glycoproteins are formed by successive transfer on dolichol-P-P-GlcNAc and that once they are formed, transfer to protein occurs (6,7). This theory does not exclude the further addition of other monosaccharides to the glycoprotein.

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